T-cell Differentiation in Chronic Hepatitis C Being Treated with Pegylated Interferon α+Ribavirin

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ABSTRACT

Objective : We evaluated the proportion of CD4+ and CD8+ T-cell subsets (naïve T [NT], central memory T [CMT] cells, effector memory T [EMT] cells, and terminally differentiated T [TDT] cells) in patients with chronic hepatitis C virus (HCV) infection, with special reference to the outcome of antiviral therapy, to better understand the relationship between the immune response and the eradication of HCV.

Materials and Methods : A total of 37 patients (12 men, 25 women ; median age [age range], 61 [19-74] years) with chronic HCV genotype 1b (G1b) infection were treated with pegylated interferon αIIb and ribavirin in our hospital from 2007 though 2009. We examined serial changes in the percentages of T-cell subsets in the peripheral blood at 3 time points (before treatment, week 12 of treatment, and the end of treatment).

Results : The percentages of CD4+ and CD8+ EMT cells decreased significantly after 12 weeks of treatment in patients who were sustained virological responders or transient virological responders. In addition, percentages of CD4+ EMT cells and CD8+ CMT cells decreased significantly by the end of treatment in sustained virological responders.

Conclusion : Changes in the percentages of T-cell subsets may contribute to the virological effect of treatment with pegylated interferon αIIb and ribavarin, and this knowledge may enhance efforts to develop novel antiviral therapies. (Jikeikai Med J 2011 ; 58 : 45-55)

Key words : hepatitis C virus, T-cell differentiation, pegylated interferon αIIb and ribavirin

INTRODUCTION

The spread of hepatitis C virus (HCV) is a serious public health problem, with an estimated 200 million chronically infected patients worldwide[1-3]. By itself, HCV is not cytopathic ; nevertheless, antigen-specific cellular immune responses against HCV-infected host hepatocytes causes hepatitis and apoptosis of hepatocytes[3]. If the immune response is sufficient, HCV is eradicated, but if the immune response is insufficient, the virus will not be eliminated, and inflammation will be long lasting. Chronic HCV (CHC) infection often induces chronic hepatitis, which may lead to hepatic cirrhosis and hepatocellular carcinoma over a period of 20 to 30 years[1,2].

In the primary immune response, unprimed naïve T (NT) lymphocytes are activated and then differentiate into effector cells that eliminate infected cells (in case of first infection) and then become memory T cells (primed

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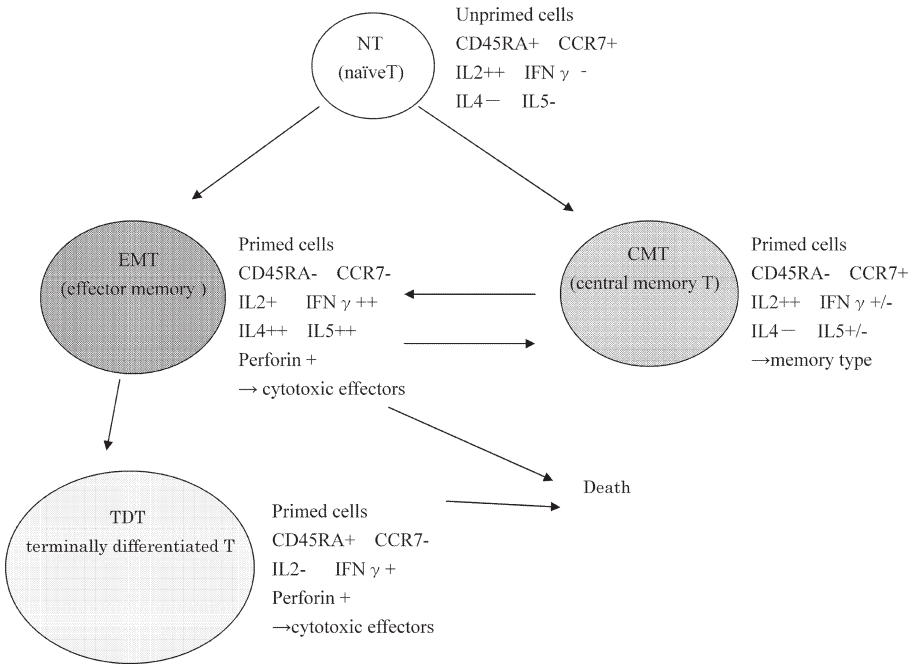


Fig. 1. Model of human T cell differentiation. In the primary response, unprimed naïveT (NT) lymphocytes are activated and then differntiate into effector cells that eliminate infected cells and then become memory T cells (primed cells). A certain percentage of the primed T cells persist as circulating memory cells that can confer protection upon secondary challenge. NT cells have more proliferative capacity, homing capacity and production of IL2 than others. CMT cells (memory type) have moderate homing capacity and mild effector function. The effector function of EMT cells and TDT cells (late EMTs, cytotoxic effectors) are stronger than those of NT cells or CMT cells, and EMT cells and TDT cells have a greater capacity to produce IFNγ, IL4, IL5 and perforin.

cells)[4-6]. A certain percentage of the primed T cells persist as circulating memory cells that can confer protection upon secondary challenge and provide a qualitatively different and quantitatively enhanced response[4,5,7,8]. Thus, once primed T cells are secondarily activated, they proliferate vigorously and generate effector cells that can migrate to B-cell areas or to inflamed tissues[7]. Most T cells in the peripheral blood are classified as either CD4+ or CD8+ cells and can be subclassified on the basis of their level of differentiation and their roles in the primary and secondary immune responses. The level of T-cell differentiation in humans is determined by assessing functional characteristics, and cells can be classified into 1 of 4 distinct cellular subsets (Fig. 1)[4-10]: NT cells, central memory T cells (CMT cells), effector memory T cells (EMT cells), and terminally differentiated T cells (TDT cells).

Many markers are expressed on the surfaces of T cells, and their roles in the function and differentiation of T lymphocytes have been extensively investigated[4-11]. Among these surface markers, CCR7 and CD45RA are generally used as markers of T-cell differentiation.

CD45RA has been widely used as a marker of NT lymphocytes[5] and is highly expressed on TDTs (late EMTs). Chemokine receptor CCR7-positive memory cells express lymph-node—homing receptors and lack immediate effector function[7,8], whereas CCR7-negative memory cells express receptors enabling them to migrate to inflamed tissues, where they display immediate effector functions. The T-cell subsets are thus determined according to the expression of CCR7 and CD45RA as follows: NT cells (unprimed cells) are both CD45RA+ and CCR7+; CMT cells are CD45RA− and CCR7+; EMT cells are both CD45RA− and CCR7−; and TDT cells are CD45RA+ and CCR7−[5,7,8]. The effector functions of EMT cells and TDT cells are stronger than those of NT cells or CMT cells, and EMT cells and TDT cells have a greater capacity to produce interferon (IFN)-γ, interleukin (IL)-4, IL-5 and perforin[6,7,9].

In cases of acute HCV infection, the virus is spontaneously eradicated in only 16% to 50% of patients, and CHC infection develops in the remaining patients. IFN is a key element of the innate immune response against acute viral

infections[3,12] and is extremely important for the elimination of HCV. The rate of successful elimination with IFN in patients with prolonged acute HCV infection is 71% to 94%[13]. However, in patients with CHC infection, achieving a sustained virological response (SVR) with IFN monotherapy is difficult[14], especially for infections involving HCV genotype 1 (G1). The introduction of a combined treatment involving pegylated (PEG) IFNα and ribavirin has increased the SVR rate to about 50%, even in cases of CHC G1 infection[14,15].

In the present study, we analyzed the changes in 4 T-cell subsets in the peripheral blood of patients receiving PEG-IFNαIIb and ribavarin for CHC G1b infection. We also examined whether these changes correlate with the virological effect of the treatment.

Materials and Methods

Patients

The study involved 37 patients with CHC (12 men, 25 women ; median age [age range] of 61 [19-74] years) and HCV G1b infection undergoing treatment with PEG-IFNαIIb (Pegintron® ; Merck Sharp & Dohme, Japan) and ribavirin (Rebetol® ; Merck Sharp & Dohme, Japan) in our hospital from 2007 through 2009. Written informed consent was obtained from each subject before blood was drawn. This study was approved by the institutional review board of The Jikei University School of Medicine. Blood samples were collected 3 times : at the time of hospital admission (before treatment), at week 12 of therapy, and at the end of therapy (week 48).

Detection of HCV-RNA

The quantity of HCV-RNA in the peripheral blood was determined with the TaqMan® Real-time RT-PCR assay (Roche Diagnostics, Basel, Switzerland). The lower limit of quantitative detection was 1.2 $\log_{10}$ IU/ml.

Liver biopsy

Liver biopsy was performed in 32 patients. The New Inuyama scoring system was used to assess the stage of fibrosis as follows : 0=no fibrosis, 1=fibrous portal expansion, 2=bridging fibrosis, 3=bridging fibrosis with lobular degeneration, and 4=cirrhosis[16].

Treatment protocol

According to Japanese standard therapy for CHC[17], PEG-IFNαIIb was administered subcutaneously once a week, and ribavirin was administered orally every day. The dose of PEG-IFNαIIb was 1.5 μg/kg/day. Ribavirin was administered at a dosage adjusted according to body weight (600 mg/day for <60 kg, 800 mg/day for 60 to 80 kg, 1,000 mg/day for >80 kg). In each patient, treatment was continued for 48 weeks.

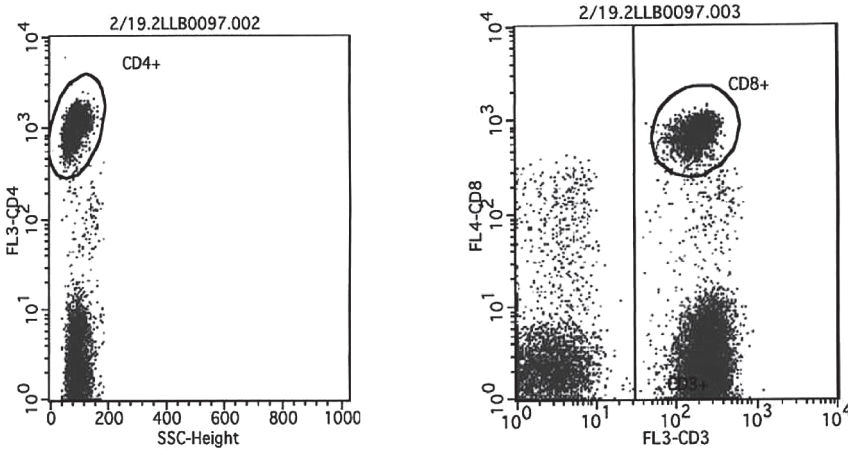
Classification of patients according to the virological response to treatment with PEG-IFNαIIb and ribavirin

Patients were divided into 3 groups on the basis on the virological response : 1) patients showing a SVR indicated by HCV-RNA remaining negative in serum during the first 6 months after the completion of therapy ; 2) transient virological responders (TVRs), in whom HCV-RNA became negative at the end of therapy and reappeared thereafter ; and 3) nonvirological responders (NVRs), in whom HCV-RNA was positive even during treatment.

Detection of circulating T lymphocyte differentiation using flow cytometry

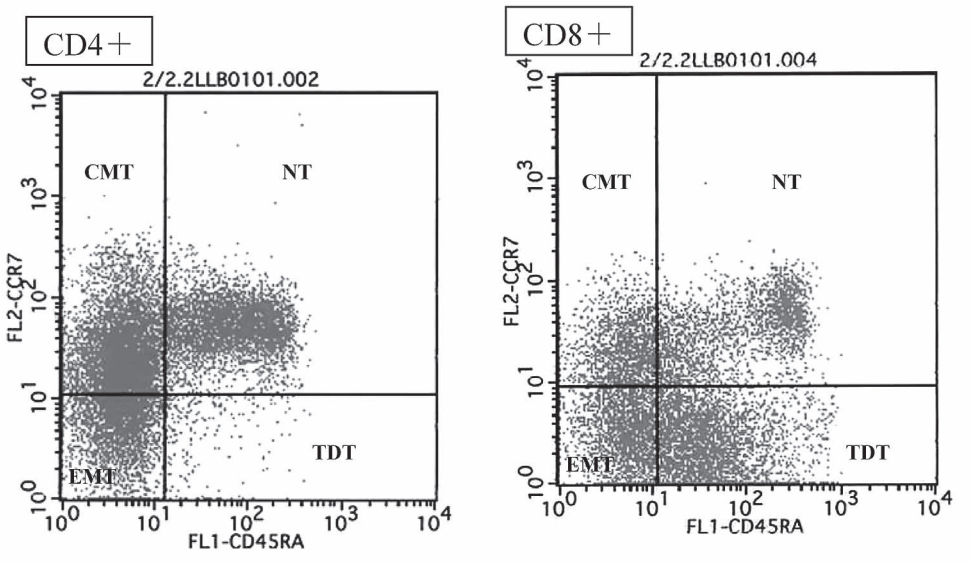
Differentiation in peripheral blood T lymphocytes was characterized by means of flow cytometry with CD45RA and CCR7 monoclonal antibodies. Three-color flow cytometric analysis was used to detect circulating CD4+ T-cell differentiation, and 4-color analysis was used to detect CD8+ T cell differentiation . Monoclonal fluorescence-labeled antibodies used in the study were as follows : phycoerythrin-cyanin (Pe-Cy5)-labeled anti-CD4 (Beckman Coulter, Fullerton, CA, USA), allophycocyan-labeled anti-CD8 (Beckman Coulter), fluorescein isothiocyanate (FITC)-labeled CD45RA (Beckman Coulter), and phycoerythrin (PE)-labeled anti-CCR7 (R&D Systems, Minneapolis, MN, USA). In addition, we used peridinin chlorophyll protein (PerCP)-labeled anti-CD3 antibody (BD Biosciences Pharmingen, San Diego, CA, USA) to distinguish T lymphocytes in the analysis of CD8+ T-cell differentiation because the number of CD8+ T cells is small in peripheral blood, and contamination of the CD8+ fraction with non-T cells could have a considerable influence on detection (Fig. 2).

To 3 ml of heparinized blood, 0.1 ml of each fluorescently labeled monoclonal antibody in 0.2% bovine serum albumin (BSA)-phosphate buffered saline (PBS) was added,



a b

Fig. 2. a : CD4+ T cells were identified by flow cytometry (circled area). b : CD8+ T cells were identified with two-color (CD3 and CD8) flow cytometry (circled area).



a b

Fig. 3. a : CD4+ T cells were identified by CD45RA and CCR7 as follows : naive T cells (NT) (CD45RA+, CCR7+), central memory T cells (CMT) (CD45RA − CCR7+), effector memory T cells (EMT) (CD45RA − CCR7 −), terminal differentiated T cells (TDT) (CD45RA+, CCR7 −). b : CD8+ T cells were identified by CD45RA and CCR7 as in Figure 2a.

gently mixed, and incubated for 30 minutes at 4°C in the dark. Next, 2 ml of lysis solution (0.83% NH_4Cl in PBS) was added to lyse the red blood cells. Following centrifugation (1,500 rpm, 400 to 450 × *g* for 5 minutes) at room temperature, the supernatant was removed, and the pellet was resuspended in PBS. These steps (addition of lysis solution, centrifugation, removal of supernatant, resuspension of pellet) were then repeated, and the pellet was washed with PBS, resuspended in 0.1 ml of PBS, and analyzed with flow cytometry (FACSCalibur ; BD Biosciences Immunocytometry Systems, San Jose, CA, USA) to determine the percentages of NT, CMT, EMT, and TDT cells among the CD4+ and CD8+ T lymphocytes (Fig. 3).

Statistical analysis

Differences between SVR, TVR, and NVR patients

were evaluated with the Mann-Whitney U-test and the χ^2 test. Sequential changes in the percentages of T-cell subsets in patients belonging to the SVR, TVR, and NVR groups who were being treated with PEG-IFN and ribavirin were evaluated with the Wilcoxon signed-rank test. Correlation analyses were performed with the Spearman correlation test. All P values were two-tailed, and the level of significance was set at $P<0.05$.

Results

Outcome of treatment with PEG-IFNαIIb and ribavirin

Of the 37 patients, 21 (57%) were classified as SVRs, 10 (27%) as TVRs, and 6 (16%) as NVRs. The characteristics of each group are shown in Table 1. SVR patients tended to be younger than TVR patients and to have higher platelet counts. The fibrosis score was higher in NVR patients than in SVR patients, and the rate of cirrhosis was higher in the SVR group. There were no differences between the groups with respect to sex, body mass index (BMI), alanine aminotransferase (ALT), and HCV-RNA level at the start of treatment.

Relationship between T cell-differentiation at the time of entry and clinical background (Table 2)

Age was negatively correlated with the percentages of CD4+ NT cells and CD8+ NT cells but was positively correlated with the percentages of CD4+ CMT cells and CD8+ TDT cells. The HCV-RNA level was positively correlated with the percentage of CD4+ EMT cells. The fibrosis score was positively correlated with the percentages of CD4+ CMT cells and CD8+ CMT cells. In our study, CD4+ TDT cells were not investigated because this subset was too small to evaluate in detail.

Differences in the proportion of T cell subsets according to the outcome of treatment (SVR, TVR and NVR) at the time of entry, at week 12 of treatment, and at the end of treatment

There was no difference in the percentages of CD4+ and CD8+ T-cell differentiation between the 3 groups (SVR, TVR, NVR) at the time of entry, at week 12 of treatment, or at the end of treatment.

Sequential changes in CD4+/CD8+ T cell subsets during treatment with PEG-IFN and ribavirin in SVR patients

Compared with those before treatment, the percentages of CD4+ and CD8+ NT cells after treatment with PEG-IFN and ribavirin were significantly higher, whereas the

Table 1. Characterics of therapy groups. Data are expressed as the median (range). ALT: alanine aminotransferase, PLT: platelet count, Fibrosis score: fibrosis score was evaluated according to the New Inuyama Scoring System. *Age of SVR group was lower than the TVR group ($P<0.05$). **PLT of SVR group was higher than the TVR group ($P<0.05$). ***The average fibrosis score of the SVR group was lower than that of the NVR group ($P<0.05$).

	SVR ($n=21$)	TVR ($n=10$)	NVR ($N=6$)
Age (years)	59 (19-72)	63.5 (51-70)*	65 (52-74)
Sex (male/female)	5/16	4/16	3/3
BMI (kg/m^2)	22.24 (18.36-28.94)	24.24 (18.86-26.44)	22.75 (19.75-31.01)
ALT (IU/ml)	44 (14-206)	37 (22-116)	75 (22-132)
PLT ($\times10^4$/ml)	20.8 (10.3-41.3)	13.95 (10.8-25)**	15.75 (10.3-19.8)
HCVRNA (log IU/ml)	6.3 (3.6-6.9)	6.3 (5.9-6.6)	6.3 (2.3-6.2)
Fibrosis score ($n=32$)			
F0	2	1	0
F1	8	4	1
F2	3	3	0
F3	2	2	1
F4	1	0	4***

Table 2. Correlation between clinical background and T cell differentiation before therapy (n=37). *Negative relationship between clinical background and T cell differentiation (P<0.05). **Positive relationship between clinical background and T cell differentiation (P<0.05).

	CD4+NT	CD4+CMT	CD4+EMT	CD8+NT	CD8+CMT	CD8+EMT	CD8+TDT
Age	−0.45*	0.43**	0.17	−0.55*	0.22	0.02	0.51**
BMI	0.24	0.03	0.20	0.00	−0.08	0.00	0.03
ALT	0.01	−0.08	−0.03	−0.03	0.15	−0.18	0.01
HCVRNA	−0.09	−0.04	0.34**	0.00	0.06	0.27	−0.04
Fibrosis score	−0.28	0.37**	−0.12	0.06	0.55**	−0.16	0.26

percentages of CD4+ EMT cells and CD8+ EMT/TDT cells were significantly lower at week 12 of treatment (Fig. 4a). At the end of treatment, the percentage of CD4+ NT cells was significantly higher and the percentage of CD4+ EMT cells was significantly lower. In addition, while the percentage of CD8+ EMT cells showed no significant change at the end of treatment, the percentage of CD8+NT cells was significantly higher and that of CD8+CMT cells was significantly lower.

Sequential changes in CD4+/CD8+ T cell subsets during treatment with PEG-IFN and ribavirin in TVR patients

At week 12, the percentages of CD4+ and CD8+ NT cells were significantly higher and those of CD4+ and CD8+ EMT cells and CD8+ TDT cells were significantly lower (Fig. 4b). At the end of treatment, the percentage of CD4+ NT cells was marginally higher and that of CD4+ EMT cells was marginally lower. In addition, the percentage of CD8+ NT cells was significantly increased, and the percentage of CD8+ TDT cells was significantly decreased. Treatment with PEG-IFN and ribavirin had no effect on the proportion of CD8+ EMT cells in TVR patients.

Sequential changes in CD4+/CD8+ T cell subsets under treatment with PEG-IFN and ribavirin in NVR patients

As shown in Fig. 4c, after 12 weeks of treatment with PEG-IFN and ribavirin the percentage of CD8+ NT cells was higher in NVR patients, whereas that of CD8+ TDT cells was significantly lower. At the end of treatment, the percentage of only CD4+ NT cells was significantly increased ; the percentages of all other subsets were unchanged.

DISCUSSION

In the present study, we examined sequential changes in CD4+ and CD8+ T-cell subsets in patients with CHC treated with PEG-IFN and ribavirin and compared these changes with the virological effects. Our initial finding that the pattern of T-cell differentiation changes with age (especially the decrease in CD4+ and CD8+ NT cells) observed in patients with CHC may represent a physiological change related to thymic involution associated with aging, as has been shown to occur in healthy persons[18,19]. In addition, our observations that the level of HCV-RNA positively correlates with the percentage of CD4+ EMT cells and that the fibrosis score positively correlates with the percentages of CD4+ and CD8+ CMT cells suggest that T-cell differentiation plays a role in the development of chronic liver disease in HCV infection.

However, we found that no difference in the percentages of CD4+ and CD8+ subsets between SVR, TVR, and NVR patients at any time, including during treatment. Our data conflict with those of Lee et al.[20] who have reported that the percentages of CD4+ EMT cells were higher in patients who did not show a SVR than in patients who showed a SVR, even before treatment. This discrepancy could be due in part to differences in patient background. The patients in the study of Lee et al. were considerably younger, the HCV genotype was not set at G1b, and the serum HCV-RNA level was lower. These factors may have affected the percentages of T-cell subsets present before treatment. Our finding that serum HCV-RNA levels positively correlate with the percentage of CD4+ EMT cells is interesting because EMT cells play a major role in HCV recognition and elimination. However, Shen et al.[21] have reported a decrease in CD4+ NT cells and in-

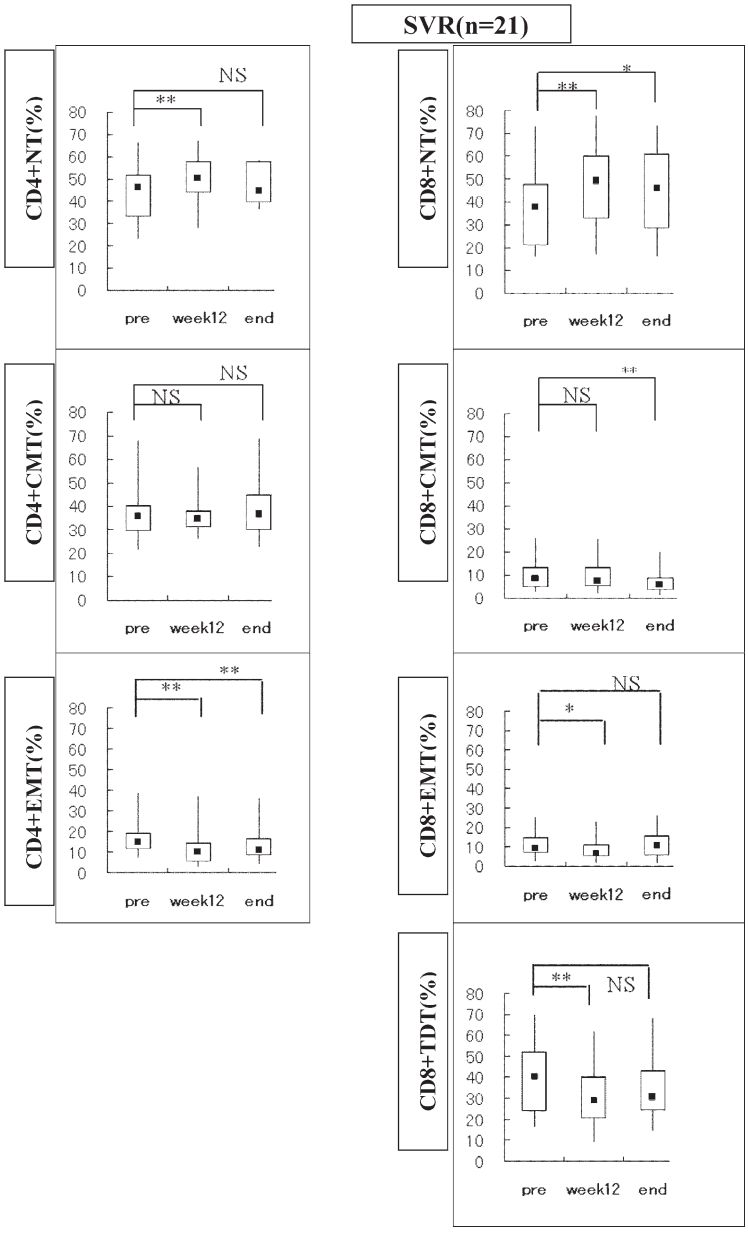


Fig. 4a

creases in CD4+ CMT cells and CD4+ EMT cells in patients with CHC in which CD127 expression was lower in these T-cell subsets ; they suggested that these changes are associated with a less efficient immune response against HCV in patients with CHC infection. The expansion in CD4+ EMT cells we observed in patients with CHC may be associated with the persistence of HCV rather than its eradication.

At week 12 of treatment with PEG-IFN and ribavirin, the percentages of CD4+/CD8+ NT cells had increased, while those of CD4+/CD8+ EMT cells had decreased in SVR and TVR patients, but these changes were not observed in NVR patients. This finding may be related to a reduction in the amount of virus in the serum, because HCV-RNA had decreased to an undetectable level by the 12th week of treatment in most SVR and TVR patients but was still detectable at 12 weeks in NVR patients. As discussed earlier, the percentage of CD4+ EMT cells before treatment in patients with CHC positively correlated with the serum level of HCV-RNA, and, therefore, a decrease in

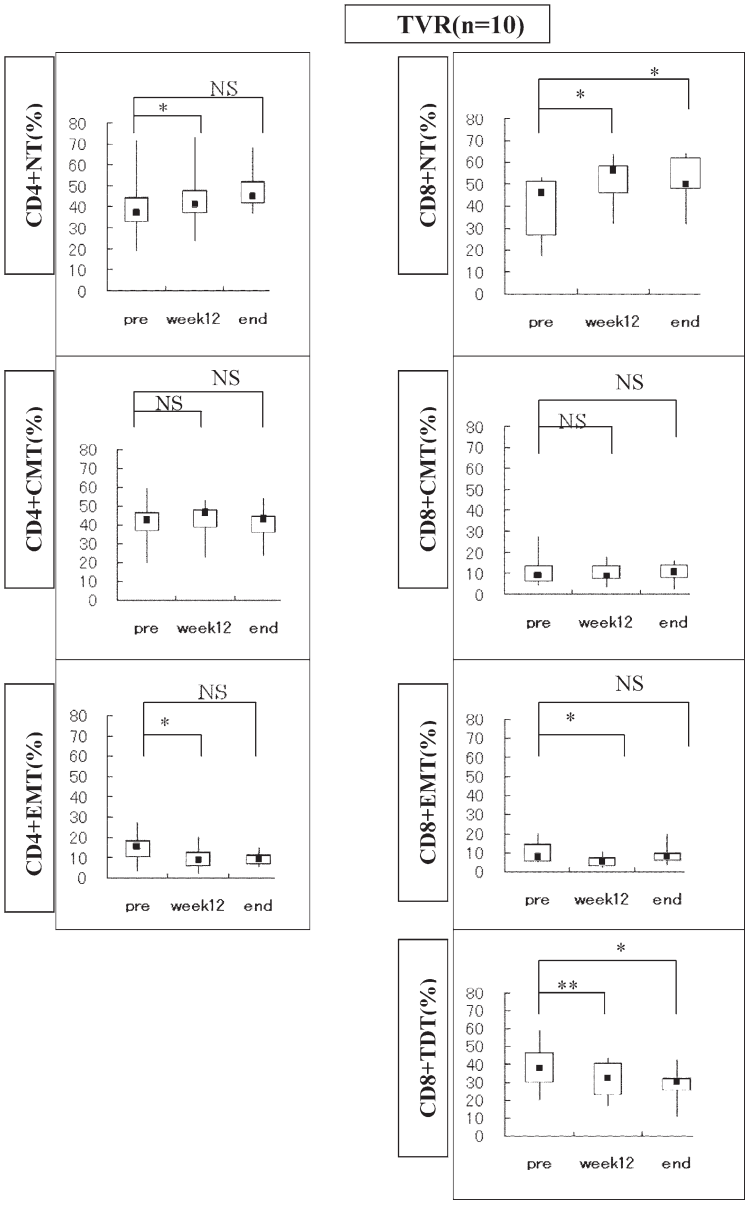


Fig. 4b

CD4+ EMT cells might indicate that the chronic HCV infection is being eliminated. In general, as CD4+ T cells are required to maintain CD8+ T cells in chronic infection [6,11], a reduction in the percentage of CD4+ EMT cells might contribute to decreases in CD8+ EMT and CD8+ TDT cells, as was observed in SVR patients and TVR patients in our study. This finding is consistent with the report of Lee et al.[20], who have reported that percentages of CD4+ EMT and CD8+ EMT/TDT cells decreased with treatment with PEG-IFN and ribavirin (3 to 10 months) in SVR patients. Concerning CD8+ EMT cells in relation to chronic HCV infection, Francavilla et al.[22] have reported that virus-specific CD8+ EMT cells are functionally obliterated and that this dysfunction is critical for the establishment of HCV persistence in acute HCV infection. They found that although HCV-specific CD8+ CMT cells could efficiently proliferate and differentiate, the large population of CD8+ EMT cells in the peripheral blood was functionally compromised and produced IL-2 production at low levels. Although we examined neither IL-2 production nor the expression of specific surface markers related to cell function, our results strongly suggest that a significant number of

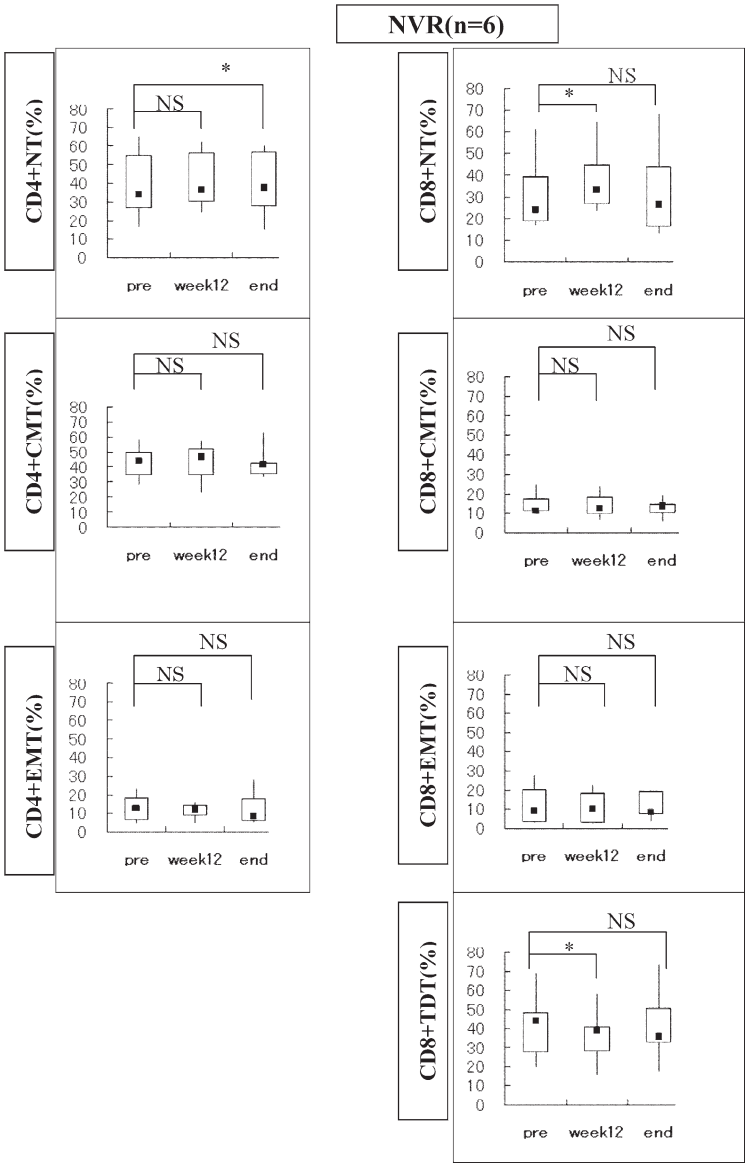


Fig. 4c

Fig. 4. Changes in the proportion (%) of T cell subsets with therapy in three groups (a-SVR, b-TVR and c-VR). Data are presented as box- and whisker-plots showing the median, 25th and 75th percentiles, and range. $*P<0.05$, $**P<0.01$, NS : Not Significant. a : Changes in the proportion (%) of T cell subsets with therapy in SVR patients ($n=21$). CD4+ and CD8+NT cells increased, while CD4+EMT, CD8+EMT, and CD8+TDT cells (%) decreased at week 12. At the end of therapy CD4+EMT and CD8+CMT cells decreased, while CD8+NT cells increased. b : Changes in the proportion (%) of T cell subsets with antiviral therapy in TVR patients ($n=10$). CD4+NT and CD8+NT cells increased, but CD4+EMT, CD8+EMT, and CD8+TDT cells decreased at week 12. At the end of therapy, CD8+NT cells increased, while CD8+TDT cells decreased. c : Changes in the proportion (%) of T cell subsets with antiviral therapy in NVR patients ($n=6$). There was no significant change in the proportion of CD4+T cells, but CD8+NT cells increased and CD8+TDT cells decreased at week 12. At the end of therapy, CD4+NT cells increased, but no significant changes were observed in the proportion of other cells.

functionally distorted HCV-specific CD4+ EMT and CD8+ EMT/TDT cells were eliminated in SVR and TVR patients after 12 weeks of therapy.

At the end of treatment, the percentage of each T-cell subset was similar to that observed at the 12th week of therapy, except for the significant decrease in the percent-

age of CD8+ CMT cells observed in SVR patients. In contrast, the percentages of CD8+ CMT cells either did not change or even increased in NVR and TVR patients. Although we cannot fully explain its underlying mechanism and significance, the reduction in CD8+ CMT cells we observed in SVR patients may be related to the resolution of fibrosis due to the cessation of inflammation after the complete elimination of HCV. Patients with advanced liver fibrosis are at greater risk for being exposed to novel antigens that convert NT cells into CMT cells, and this may explain the positive correlation between the fibrosis score and the percentage of CD8+ CMT cells we observed. Thus, the decrease in CMT cells observed in SVR patients may be a hallmark of the resolution of liver fibrosis.

Our study has several limitations. First, our study included only 6 NVR patients. Second, we could not evaluate intrahepatic T cells, which are thought to be critical for immunity to HCV[11,23,24]. Finally, we did not identify HCV-specific EMT cells to examine their function after stimulation with HCV antigens (for example, core protein, NS3/4, and NS5A). Additional studies that will overcome these limitations are urgently needed to elucidate the significance of changes in T-cell subsets resulting from treatment with PEG-IFN and ribavirin.

In conclusion, our data indicate that a decrease in the percentage of CD4+ EMT cells after 12 weeks of treatment with PEG-IFN and ribavirin is a hallmark of a favorable antiviral response in patients with CHC. This change may be caused by an extreme reduction in viral load and a reduction in the number of dysfunctional HCV-related CD4+ EMT cells that would otherwise increase the duration of HCV infection. The decrease in CD8+ CMT cells observed at the end of treatment in SVR patients might be related to the resolution of fibrosis resulting from a cessation of inflammation.

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